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(54) Title: INSULIN MIMETIC AND ENHANCER ASSAY

(57) Abstract

Compounds that are insulin mimetic and enhancer agents and methods of identifying compounds that mimic and enhance insulin action are provided.

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TITLE OF THE INVENTION

INSULIN MIMETIC AND ENHANCER ASSAY

CROSS-RELATED TO OTHER APPLICATIONS

This is a continuation-in-part of U.S. Serial No. 08/414,976 filed March 31, 1995, now pending.

BACKGROUND OF THE INVENTION

The invention relates to insulin mimetic and enhancer agents and more specifically to methods of identifying compounds that mimic and enhance insulin action and compounds identified by the method.

Insulin is a hormone that is necessary for normal carbohydrate, protein, and fat metabolism in mammals. People with insulin-dependent (Type I) diabetes mellitus do not produce enough insulin of this hormone to sustain life and, therefore, depend on exogenous insulin for survival. In contrast, individuals with non-insulin-dependent (Type II) diabetes are not dependent on exogenous insulin for survival. However, over time, many of individuals with type II diabetes show decreased insulin production, which requires supplemental insulin for adequate blood glucose control, especially during times of stress or illness. An exogenous insulin regimen is often required in the treatment of secondary diabetes, i.e., diabetes occurring in relation to other disease states such as pancreatic disease. Insulin is also used in some cases of gestational diabetes to obtain optimum blood glucose control. The conventional route of insulin administration is via a needle and syringe. Continuous subcutaneous insulin infusion with an infusion pump is an alternative to conventional injection therapy for achieving normalized levels of blood glucose.

Insulin initiates its metabolic and growth promoting effects upon binding to its tetrameric receptor. The binding activates a kinase in the beta -subunit, which catalyzes the intramolecular autophosphorylation of specific tyrosine residues of its own β-subunits. Autophosphorylation enhances tyrosine kinase activity toward other

protein substrates. Considerable evidence suggests that insulin receptor tyrosine kinase activity is essential for many, if not all of the biological effects of insulin. However, the precise biochemical mechanisms linking receptor kinase-mediated tyrosine phosphorylation to the regulation of cellular metabolic pathways are not completely defined.

The instant invention is a novel assay to identify insulin mimetic and enhancer compounds. The assay is designated as the Insulin Mimetic and Enhancer Assay. The purpose of the IMA-II is to discover nonpeptide, small molecules that may be developed into insulin mimetic and enhancer agents. The assay is based on insulin receptor tyrosine phosphorylation, and activation of the receptor tyrosine kinase events immediately downstream of insulin stimulation. In brief, Chinese Hamster Ovary (CHO) cells expressing human insulin receptor are plated and treated with insulin or test agents. CHO.T cells are one type of CHO cells that express human insulin receptor. The treated cells are lysed, and the insulin receptor is purified. The level of tyrosine phosphorylation of the receptor is determined using an anti-phosphotyrosine antibody conjugated to alkaline phosphatase and its chromogenic substrate. The insulin receptor tyrosine kinase activity (IRTK) is determined using an exogenous substrate and γ-^{32}P-ATP. The assay facilitates rapid screening and high throughput of test compounds.

SUMMARY OF THE INVENTION

The invention relates to insulin mimetic and enhancer and enhance agents and more specifically to methods of identifying compounds that mimic and enhance insulin action and compounds identified by the method.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the scheme of insulin mimetic and enhancer assay.

Figure 2 shows the activation of IRTK by insulin in CHO.T cells.

Figure 3 shows the activation of IRTK by a natural product (N-400306).

Figure 4 shows the enhancement of insulin stimulation of IRTK by a natural product (N-400306).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to insulin mimetic and enhancer agents and more specifically to methods of identifying compounds that mimic and enhance insulin action and compounds identified by the method. More specifically, the instant invention is a novel assay to identify insulin mimetic and enhancer compounds. The assay is designated as the Insulin Mimetic and Enhancer Assay. The purpose of the assay is to discover nonpeptide, small molecules that may be developed into insulin mimetic and enhancing agents. The assay is based on insulin receptor tyrosine phosphorylation, and activation of the receptor tyrosine kinase events immediately downstream of insulin stimulation. In brief, Chinese Hamster Ovary (CHO) cells expressing human insulin receptor (CHO-T) are plated and treated with insulin or test agents. The treated cells are lysed, and the insulin receptor is purified. The level of tyrosine phosphorylation of the receptor is determined using anti-phosphotyrosine antibody conjugated to alkaline phosphatase and its chromogenic substrate. The IRTK activity is determined using an exogenous substrate and γ-^{32}P-ATP. The assay is also performed by treating the cells with test agents in the presence of sub-maximal concentration of insulin, in an effort to identify insulin sensitigers. The assay facilitates rapid screening and high throughput of test compounds.

An insulin-related disease, as used herein, is a disease, disorder, or condition in which some aspect of insulin expression, metabolism, or action is disrupted or, a disease in which insulin action contributes to the disease. An insulin resistant disease, as used herein, is any disease, disorder, or condition in which a normal amount of insulin results in a less than normal biological response. Examples of insulin-deficient diseases include Type I or insulin-dependent diabetes.

Examples of insulin resistant diseases include Type II diabetes, obesity, age-related insulin resistance, and insulin resistance that arises secondary to infections, hormonal disorders, or other causes.

Resistance to insulin may be present in several serious disorders, including Type II diabetes mellitus, obesity and hypertension. Resistance to insulin is manifested by reduction in the effectiveness of a given dose of insulin compared to that obtained in a non-resistant state. Thus, in an insulin-resistant patient with Type 2 diabetes mellitus, the ability of both endogenous insulin and insulin administered exogenously to control the chronic hyperglycemia suffered by such patients is seriously compromised. In both Type I and Type II diabetic patients, uncontrolled hyperglycemic symptoms result in complications such as premature atherosclerosis, intercapillary glomerulosclerosis, retinopathy, neuropathy and kidney failure.

The invention includes a method of assaying an effect of a therapeutic agent on activation of insulin signal transduction in cells. The method includes administering the agent to a test organism, e.g., a cultured cell or a mammal, and measuring the effect of the drug on an aspect of insulin receptor metabolism, e.g., measuring the level of the insulin receptor phosphorylation. A change in an aspect of insulin receptor metabolism indicates an effect of the agent.

The invention includes a method of assaying an effect of a therapeutic agent which alters the ability of a tyrosine kinase to phosphorylate a substrate. The method includes administering the drug to a test organism, e.g., a cultured cell or a mammal, and measuring the level of phosphorylation of a substrate.

The invention further includes a method of treating a mammal e.g., a human, suffering from a disease caused by insulin deficiency or insulin resistance, e.g., Type I and Type II diabetes mellitus. The method includes administering to the mammal a therapeutically effective amount of a therapeutic agent, e.g., agent which alters an aspect of the metabolism of insulin receptor, e.g., the level of insulin receptor phosphorylation, e.g., by increasing the activity of a kinase or decreasing the activity of a phosphatase.

Methods of the invention also allow the treatment of a variety of diseases, e.g., insulin related diseases, insulin resistant diseases, diseases characterized by abnormal cellular proliferation, and diseases caused by the phosphorylation of a substrate by a tyrosine kinase, by intervening in aspects of insulin receptor metabolism.

The present invention provides methods of identifying compounds that interact with insulin receptor. Methods of identifying compounds are exemplified by an assay, comprising:

(a) incubating Chinese Hamster Ovary cells that express human insulin receptor with a solution containing a test compound alone, or in the presence of submaximal concentration of insulin;

(b) measuring tyrosine kinase activity; and

(c) comparing the tyrosine kinase activity of the mixture to a standard.

The present invention is also directed to methods for screening for compounds which modulate the expression of DNA or RNA encoding genes regulated by insulin or which modulate the function of insulin regulated proteins. Compounds which modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding genes regulated by insulin or the function of insulin regulated protein. Compounds that modulate the expression of DNA or RNA encoding genes regulated by insulin or the function of insulin regulated protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample.

Kits containing the compounds that modulate DNA, RNA, antibodies to insulin regulated protein or insulin regulated protein may be prepared. Such kits are useful for a variety of purposes including but not limited to forensic, taxonomic or epidemiological studies.

Pharmaceutically useful compositions comprising modulators of insulin activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration.

The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in

such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

The CHO.T cells of the present invention are available from Dr. Richard Roth, Stanford University; alternatively, cell lines similar to the CHO.T cells used herein may be prepared by one skilled in the art. For example, NIH3T3 cells, COS cells, Rat-1 cells and other appropriate fibroblasts transfected with cDNA encoding human insulin receptor can also be used in this assay.

The present invention relates to novel modalities for treatment of diabetes, and other diseases caused by dysfunctional signal transduction by receptor type tyrosine kinases. in particular the insulin receptor.

The present invention further relates to methods for screening and identifying compounds capable of modulating the activity of insulin receptor tyrosine kinases. Such compounds may be insulin mimetic or sensitizer, by interaction directly or indirectly with the receptor (e.g., through inhibition of insulin receptor specific tyrosine phosphatase). Such compounds may be used in the treatment of diabetes and other diseases mediated by the insulin receptor type tyrosine kinases.

SIGNAL TRANSDUCTION

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. The process is generally imitated by the binding of extracellular factors (such as hormones and growth factors) to membrane receptors on the cell surface. The biochemical pathways through which signals are transmitted within cells comprise a circuitry of directly or functionally connected interactive proteins. Each protein component in a pathway integrates signals from upstream activators and passes them onto various downstream effector proteins.

One of the key biochemical mechanism of signal transduction involves the reversible phosphorylation of tyrosine residues on proteins. The phosphorylation state of a protein may affect its conformation and/or enzymic activity as well as its cellular location.

The phosphorylation state of a protein is modified through the reciprocal actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Generally, the level of tyrosine phosphorylation increases after the cell has been stimulated by an extracellular factor. Research in this area has largely focused on protein tyrosine kinases (Sefton *et al.*, 1980 *Cell*, 20:807-16; Heldin & Westermark, 1984 *Cell*, 37:9-20; Yarden and Ullrich, 1988 *Ann. Rev. Biochem.* 57:443-78; Ullrich and Schlessinger, 1990 *Cell*, 61:203-12).

Protein tyrosine kinases comprise a large family of transmembrane as well as cytoplasmic enzymes with multiple functional domains (Taylor *et al.*, 1992 *Ann. Rev. Cell Biol.* 8:429-62). The binding of an extracellular factor or ligand allosterically transduces a signal to the inner face of the cell membrane where the cytoplasmic portion of the receptor protein tyrosine kinase (RPTKs) initiates a cascade of molecular interactions that disseminate the signal throughout the cell and into the nucleus.

Ligand-induced activation of the kinase domain and its signaling potential are mediated by receptor dimerization. Receptor dimerization stabilizes the interactions between adjacent cytoplasmic domains, and activates the intrinsic kinase activity of the receptor. Once activated, the receptor self-phosphorylates (autophosphorylation or transphosphorylation) on specific tyrosine residues in the cytoplasmic domain (Schlessinger, 1988, *Trends Biochem., Sci.* 13:443-7, Schlessinger and Ullrich, 1992, *Neuron*, 9:383-91, and references therein). In case of insulin receptor-type RPTKs, the receptor exists naturally as a dimer, undergoing a conformational change and autophosphorylation upon ligand binding.

While it is widely appreciated that these RPTKs assume a key role in signal transduction, the part played by phosphatases remains poorly understood. Like the PTKs, the protein tyrosine phosphatases (PTP) comprise a family of transmembrane and cytoplasmic enzymes. (Hunter, 1989, *Cell* 58:1013-16; Fischer *et al.*, 1991, *Science* 253:401-6; Saito & Streuli, 1991, *Cell Growth and Differentiation* 2:59-65; Pot and Dixon, 1992, *Biochem. Biophys. Acta*, 1136:35-43). It is believed that

RPTKs play a triggering role in signal transduction, while RPTPs guarantee that the trigger is reset, thereby serving to deactivate the pathway. The PTP specific for insulin receptor is not defined at present.

THE INSULIN RECEPTOR

The insulin receptor (IR) (Ebina *et al.*, 1985, *Cell*, 40, 747-758; Ullrich *et al.*, 1985, *Nature* 313:756-61) is the prototype for a family of RPTKs structurally defined as a heterotetrameric species of two α and two β subunits. Other members of the insulin receptor-type protein tyrosine kinase (IR-PTK) family include the receptor for insulin-like growth factor I (IGF-1 R; Ullrich *et al.*, 1986, *EMBO J* 5:2503-12) and the insulin related receptor (IRR; Zhang *et al.*, 1992, *J. Biol. Chem.* 267:18320-8), the ligand(s) for which are at present unknown.

Insulin binding to the insulin receptor triggers a variety of metabolic and growth promoting effects. Metabolic effects include glucose transport, biosynthesis of glycogen and fats, inhibition of triglyceride breakdown, and growth promoting effects include DNA synthesis, cell division and differentiation. It is known that some of these biological effects of insulin can be mimicked by vanadium salts such as vanadates and pervanadates. However, this class of compounds appears to inhibit phosphotyrosine phosphatases generally, and are potentially toxic because they contain heavy metal (U.S. Patent No. 5.155,031; Fantus *et al.*, 1989, *Biochem.*, 28:8864-71; Swarup *et al.*, 1982, *Biochem. Biophys. Res. Commun.* 107:1104-9).

Diabetes mellitus is a heterogeneous primary disorder of carbohydrate metabolism with multiple etiologic factors that generally involve insulin deficiency or insulin resistance or both. Type I, or juvenile onset, or insulin-dependent diabetes mellitus, is present in patients with little or no endogenous insulin secretory capacity. These patients develop extreme hyperglycemia and are entirely dependent on exogenous insulin therapy for immediate survival. Type II, or adult onset, or non-insulin-dependent diabetes mellitus, occurs in patients who

retain some endogenous insulin secretory capacity, however the great majority of them are both insulin deficient and insulin resistant. Insulin resistance can be due to insufficient insulin receptor expression, reduced insulin-binding affinity, or any abnormality at any step along the insulin signaling pathway. (Olefsky, 1988, in "Cecil Textbook of Medicine," 18th Ed., 2:1360-81).

Overall, in the United States the prevalence of diabetes is probably between 2 and 4 per cent, with Type I comprising 7 to 10 per cent of all cases. Secondary complications of diabetes have serious clinical implications. Approximately 25 per cent of all new cases of end-stage renal failure occur in patients with diabetes. About 20,000 amputations (primarily of toes, feet, and legs) are carried out in patients with diabetes, representing approximately half of the nontraumatic amputations performed in the United States. Furthermore, diabetes is the leading cause of new cases of blindness, with approximately 5000 new cases occurring each year.

Insulin is the primary mode of therapy in all patients with Type I and in many with Type II diabetes. Depending on the number of injections per day and type(s) of insulin used, the regimen can be more or less intensive. The most intensive method consists of constant insulin delivery into a subcutaneous site in the abdominal wall via an open loop delivery device consisting of a small insulin pump that must be worn by the patient essentially 24 hours a day. Oral hypoglycemic agents such as sulfonylureas are effective in Type II patients but approximately 10 to 20 percent of patients do not respond or cases to respond 12-24 months after beginning treatment.

Effective control of glucose level is difficult to achieve for prolonged periods even with the most meticulous mode of insulin therapy in the most motivated patients. Transplantation of the pancreas or islet cells, which normally produce insulin, continues to receive extensive study as a potential treatment. In addition, efforts towards developing newer and better external or implantable insulin-delivery devices integrated with a glucose sensor continues.

The present invention relates to novel modalities for treatment of diabetes, and other diseases caused by dysfunctional signal transduction by the insulin receptor (IR) class of protein tyrosine kinases. The present invention further relates to methods for screening and identifying compounds which mimic or enhance the ability of insulin to activate IRTK (insulin mimetic and enhancer assay) and thus have uses in the treatment of diabetes and other diseases.

The present invention relates to novel modalities for the treatment of diabetes, and other diseases caused by dysfunctional signal transduction by insulin receptor type protein tyrosine kinases (IR-PTKs).

The term signal transduction as used herein is not limited to transmembrane signaling, and includes the multiple pathways that branch off throughout the cell and into the nucleus. Within each individual circuit of the pathway, protein tyrosine kinases and tyrosine phosphatases carry out a series of phosphorylation and dephosphorylation steps which serve to propagate or terminate the signal. The present invention involves the use of compounds, antibodies, nucleic acid molecules or other approaches to modulate the activity of IRTK targets and, therefore, affect signal transduction.

PHARMACEUTICAL FORMULATE MODES OF ADMINISTRATION

The particular compound, antibody, antisense or ribozyme molecule that modulate the insulin receptor targets of the invention can be administered to a patient either by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s).

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic adminstration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by

intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

In addition to the active ingredients these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal inhibition of the PTP activity). Such

information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl *et al.*, 1975, in "The Pharmacological Basis or Therapeutics", Ch. 1 p1).

SCREENING ASSAYS

The cell lines that express the insulin receptor may be used to screen for molecules that modulate the tyrosine kinase activity. Such molecules may include small organic or inorganic compounds, antibodies, peptides, or other molecules that directly activate IRTK by inducing conformations charge and autophosphorylate of the receptor, or indirectly activate IRTK through inhibitor of the IR specific phosphate. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in a number of ways.

The ability of a test molecule to modulate the activity of IRTK hence signal transduction, may be measured using standard biochemical techniques. Other responses such as activation or suppression of catalytic activity, phosphorylation or dephosphorylation of other proteins, activation or modulation of second messenger production, changes in cellular ion levels, association, dissociation or translocation or signaling molecules, gene induction or transcription or translation of specific genes may also be monitored. These assays may be performed using conventional techniques developed for these purposes in the course of screening.

Ligand binding to its cellular receptor may, via signal transduction pathways, affect a variety of cellular processes. Cellular processes under the control of insulin signaling pathway may include, but are not limited to, normal cellular functions such as carbohydrate metabolism, proliferation, differentiation, maintenance of cell shape, and adhesion, in addition to abnormal or potentially deleterious processes such as apoptosis, loss of contact inhibition, blocking of differentiation or cell death. The qualitative or quantitative observation and measurement of any of the described cellular processes by techniques known in the art may be advantageously used as a means of scoring for signal transduction in the course of screening.

Various embodiments are described below for screening, identification and evaluation of compounds that interact directly or indirectly with IR, which compounds may affect various cellular processes under the control of the insulin receptor signaling pathway.

The present invention includes a method for identifying a compound which is capable of modulating insulin receptor-type protein kinase IR-PTK signal transduction, comprising:

(a) contacting the compound with IR or, functional derivatives thereof, in pure form, in a membrane preparation, or in a whole live or fixed cell;

(b) incubating the mixture of step (a) for an interval sufficient for the compound to stimulate IRTK enzymatic activity or the signal transduction;

(c) measuring the IRTK enzymatic activity or the signal transduction;

(d) comparing the IRTK enzymatic activity or the signal transduction activity to that of IR, incubated without the compound, thereby determining whether the compound stimulates or inhibits signal transduction.

IR-PTK signal transduction activity may be measured by standard biochemical techniques or by monitoring the cellular processes controlled by the signal. To assess modulation of kinase activity of the IR-PTK, the test molecule is added to a culture medium of IR expressing cells, IR is then immunopurified and IRTK activity measured. Where the test molecule is intended to mimic ligand stimulation, the assay is conducted in the absence of insulin. Where the test molecule is intended to enhance insulin activity, the test is conducted in the presence of insulin. The kinase activity is determined in the presence of ATP and a substrate and results are compared to those obtained for controls i.e., reaction mixtures not exposed to the test molecule.

Signal transduction is mimicked if the cellular processes under the control of the signaling pathway are affected in a way similar to that caused by ligand binding. Such compounds may be naturally occurring or synthetically produced molecules that could replace the administration of insulin in the treatment of diabetes.

The present invention also includes a method for identifying and isolating a nucleic acid molecule encoding a gene product which is capable of modulating IR-PTK signal transduction, comprising:

(a) introducing the nucleic acid molecule into host cells expressing IR or fragments thereof;

(b) culturing the cells so that the gene product encoded by the nucleic acid molecule is expressed in the host cells and interacts with IR or fragments thereof;

(c) measuring signal transduction activity;

(d) comparing the signal transduction to that of IR, or fragments thereof in cells without the nucleic acid molecule, thereby determining whether the gene product encoded by the nucleic acid molecule modulates IR-PTK signal transduction.

The above method may further include the step of:

(e) selecting and culturing the cells identified in step (d), recovering the nucleic acid molecule, thereby isolating the nucleic acid molecule.

By the term "nucleic acid molecule" is meant a naturally occurring or recombinantly generated nucleic acid molecule containing a nucleotide sequence operatively associated with an element that controls expression of the nucleotide sequence. An expression library may be created by introducing into host cells a pool of different nucleic acid molecules encoding different gene products. The host cells may be genetically engineered to coexpress IR and other relevant proteins. Such a gene library may be screened by standard biochemical techniques or by monitoring the cellular processes controlled by the signal. This approach is especially useful in identifying other native signaling molecules that are also involved in the signaling pathway.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

The following examples are provided to further define the invention without, however, limiting the invention to the particulars of the examples.

EXAMPLE 1

CHO.T cells, which overexpress human insulin receptor, were a gift of Dr. R. A. Roth, Stanford University. CHO.T cells (approximately 1.5 x 10^5 cells/well) were cultured in Hams F12

medium supplemented with 10% fetal calf serum, fungizone, penicillin and streptomycin. The 96-well plates were incubated for approximately 24 h at 37°C, which is when the cells reached confluency. The cells were washed with phosphate buffered saline (PBS) three times and then incubated in serum-free medium for 3 h at 37°C. Insulin or test compounds were added to the wells, and the cells were incubated for an additional 20 min at 37°C. The cells were washed three times with PBS and lysates were prepared. The lysates were transferred to a second 96 well plate. The wells of the second plate had been precoated with monoclonal anti-insulin receptor antibody. Antibody was diluted to a final concentration of approximately 4 mcg/mL in 20 mM $NaHCO_3$, pH 9.6. Approximately 50 mcL of diluted antibody solution was added to each well. The lysates were incubated for 16 h at 4°C to immunopurify the insulin receptor.

To detect the level of tyrosine phosphorylation of the insulin receptor captured on the plates, the washed plates are incubated for 5 h at 4°C with monoclonal antiphosphotyrosine antibody conjugated to alkaline phosphatase (Transduction Laboratories). The unbound antibody is removed and chromogenic substrate of alkaline phosphotase is added to the wells. Signals are detected at 405 nm with a microtiter plate reader.

The cell culture conditions, preparation of lysates, and assays are essentially those described in B. Zhang *et al.*, *J. Biol. Chem.*, Vol. 266, pages 990-996 (1991) and Zhang and Roth, *J. Biol. Chem.*, Vol. 267, pages 18320-18328, (1992).

EXAMPLE 2

Approximately 935 plant extracts were tested in the insulin mimetic assay II (IMA-II). Eight (8) active extracts from three different plants were identified and further analyzed in the insulin receptor tyrosine kinase assay. In the tyrosine kinase assay, the extracts demonstrated between 40% and 50% of the maximum activity of insulin. The EC_{50} values of the active extracts ranged from 5 mcg/mL to 40 mcg/mL.

EXAMPLE 3

Formulation of Pharmaceutical Compositions

Compounds identified by the method of Example 1 are formulated into pharmaceutical compositions according to standard methods. The compounds or pharmaceutical compositions are used either alone or in combination with other compounds or compositions for the treatment of animals (including humans) in need of treatment. Conditions requiring treatment include but are not limited to diabetes, obesity, regulation of appetite, congestive heart failure, anxiety, hypertension, cocaine withdrawal, congestive heart failure, memory enhancement, cardiac and cerebral vasospasm, pheochromocytoma and ganglioneuroblastoma, and Huntington's, Alzheimer's and Parkinson's diseases.

EXAMPLE 4

Methods of Treatment

Animals (including humans) having a condition, the condition being characterized by factors selected from altered levels of insulin or altered activities of insulin, which are treated with compounds or derivatives of compounds identified by the screening method or pharmaceutical compositions comprising the compounds or derivatives of compounds identified by the screening method.

EXAMPLE 5

CHO.T cells, which overexpress human insulin receptor, were a gift of Dr. R. A. Roth, Stanford University. CHO.T cells (approximately 1.5 x 10^5 cells/well) were cultured in Hams F12 medium supplemented with 10% fetal calf serum, fungizone, penicillin and streptomycin. The 96-well plates were incubated for approximately 24 h at 37°C, which is when the cells reached confluency. The cells were washed with phosphate buffered saline (PBS) three times and then incubated in serum-free medium for 3 h at 37°C. Insulin or test

compounds were added to the wells, and the cells were incubated for an additional 20 min at 37°C. The cells were washed three times with PBS and lysates were prepared. The lysates were transferred to a second 96 well plate. The wells of the second plate had been precoated with monoclonal anti-insulin receptor antibody. Antibody was diluted to a final concentration of approximately 4 mcg/mL in 20 mM $NaHCO_3$, pH 9.6. Approximately 150 mcL of diluted antibody solution was added to each well. The lysates were incubated for 16 h at 4°C to immunopurify the insulin receptor.

To determine the IRTK, twenty microliters of the kinase reaction mixture (50 mM Hepes, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1% Triton X-100, 1 mg/ml poly(Glu:Tyr)(4:1), 2 μ Ci of carrier-free [γ-^{32}P]ATP) was added to each well of the 96-well plates and the incubation was continued at 25°C for 40 min. The reaction was terminated by addition of 50 μl 100 mM phosphoric acid. The mixture was transferred to Multiscreen PH plates and washed. The radioactivities associated with the wells were determined using a Topcount. The IRTK activities stimulated by test agents were compared to that stimulated by insulin.

WHAT IS CLAIMED IS:

1. A method of identifying compounds that modulate insulin activity, comprising:

(a) incubating recombinant Chinese Hamster Ovary cells expressing human insulin receptor with a solution containing a test compound to form a mixture;

(b) measuring tyrosine kinase activity in the mixture; and

(c) comparing the tyrosine kinase activity of the mixture to a standard.

2. The method of Claim 1 wherein the cells are CHO.T cells.

3. Compounds identified by the method of Claim 1.

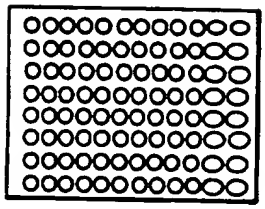
4. Pharmaceutical compositions comprising the compounds of Claim 3.

5. Compounds identified by the method of Claim 2.

6. Pharmaceutical compositions comprising the compounds of Claim 5.

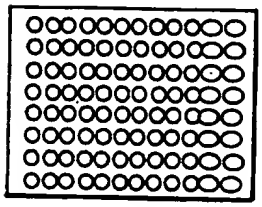
7. A method of treating an animal having a condition, the condition being characterized by factors selected from altered levels of insulin, or insulin receptor altered activities of insulin, or insulin receptor altered levels of insulin or insulin receptor activity, and combinations thereof, which comprises administration of the compounds of Claim 3 or derivatives thereof to the animal.

8. The method of Claim 7 wherein the condition selected from obesity, diabetes, anxiety, hypertension, cocaine withdrawal, congestive heart failure, memory enhancement, cardiac vasospasm, cerebral vasospasm, pheochromocytoma and ganglioneuroblastoma, Huntington's Disease, Alzheimer's Disease, Parkinson's disease, and combinations thereof.

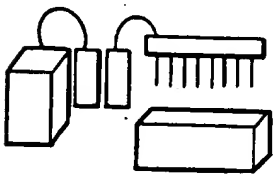


CULTURE CHO.T CELLS IN 96-WELL PLATES, 24 h

STIMULATE CELLS WITH INSULIN AND/OR TEST COMPOUNDS 20 MIN

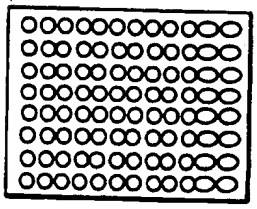


PREPARE LYSATE, IMMUNOPURIFY INSULIN RECEPTOR ON ANOTHER SET OF 96-WELL PLATES, 16h



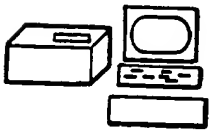
WASH PLATES

INCUBATE WITH γ-^{32}P-ATP AND SUB-STRATE, 40 MIN



TRANSFER TO MULTISCREEN PLATES

WASH PLATES



DETERMINE RADIOACTIVITY WITH TOP COUNT

FIG. 1

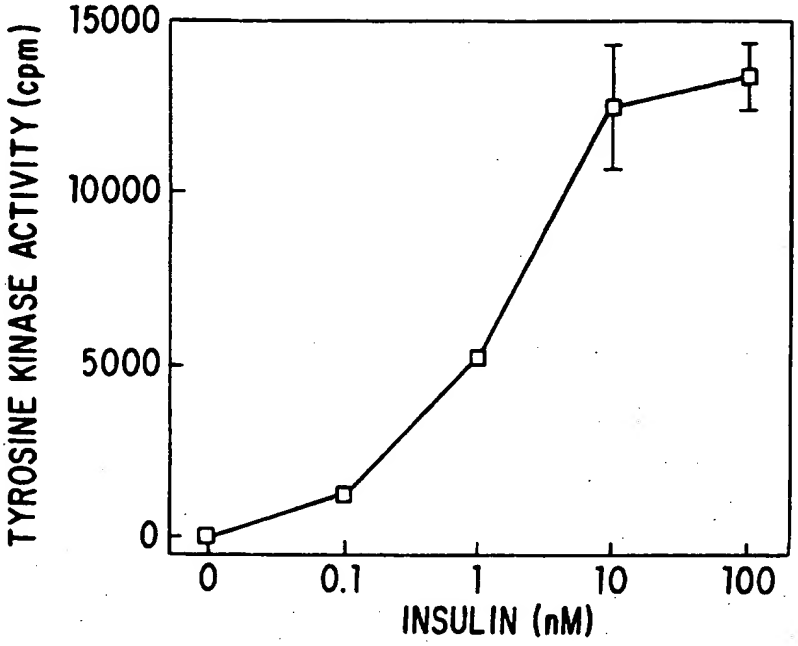

TYROSINE KINASE ACTIVITY (cpm)
15000
10000
5000
0
0
0.1
1
10
100
INSULIN (nM)

FIG. 2

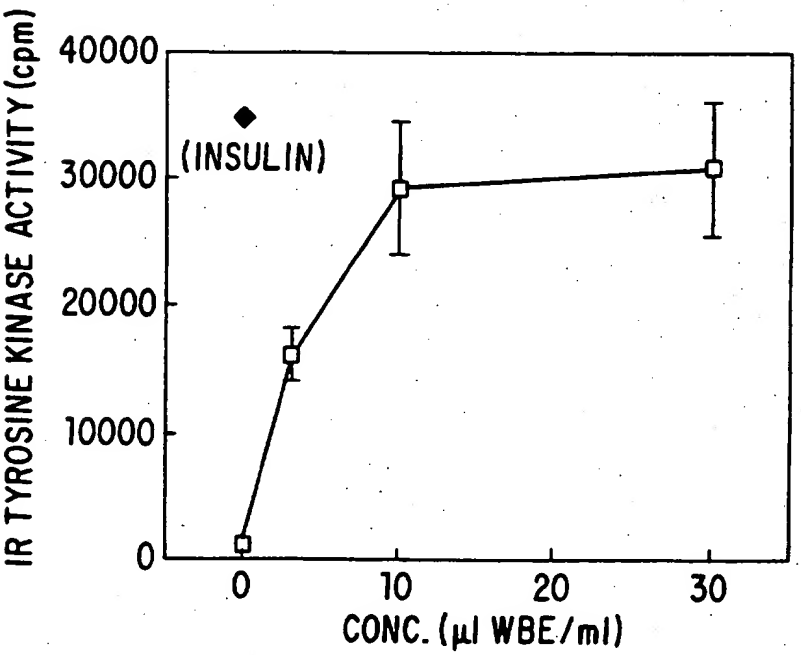

IR TYROSINE KINASE ACTIVITY (cpm)
40000
30000
20000
10000
0
(INSULIN)
0
10
20
30
CONC. (µl WBE/ml)

FIG. 3

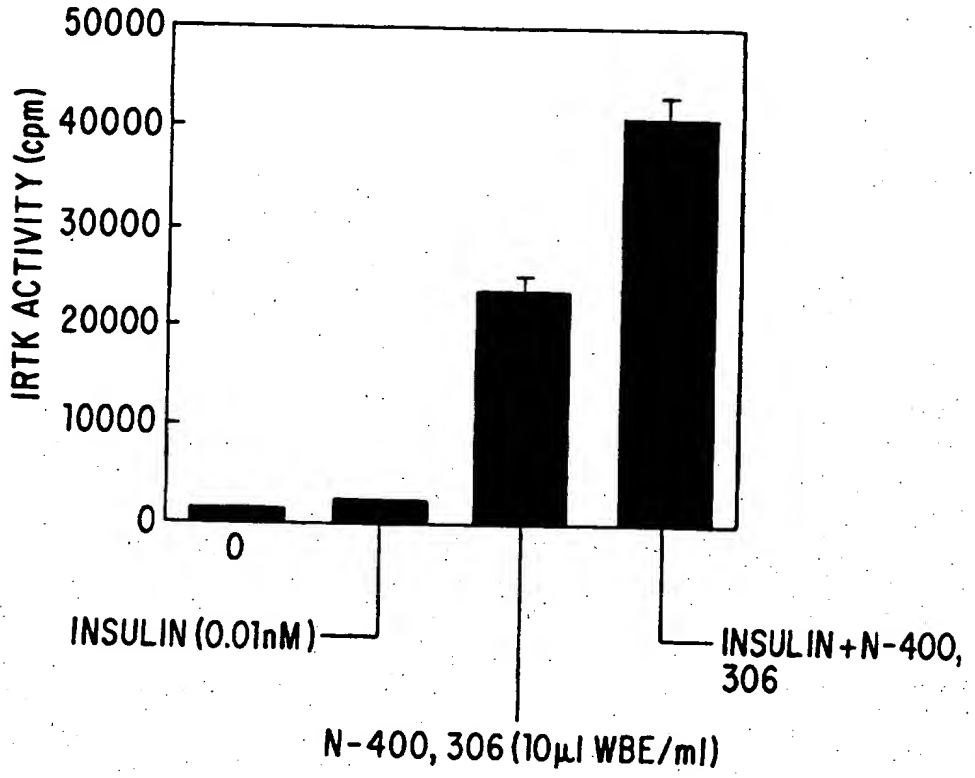

50000
40000
30000
20000
10000
0
IRTK ACTIVITY (cpm)
0
INSULIN (0.01nM)
N-400, 306 (10µl WBE/ml)
INSULIN+N-400, 306

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04226

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/567; A61K 38/28
US CL :435/7.21; 514/4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21; 514/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	The Journal of Biological Chemistry, Vol. 265, No. 16, issued 05 June 1990, G. Steele-Perkins et al., "Insulin-mimetic Anti-insulin Receptor Monoclonal Antibodies Stimulate Receptor Kinase Activity in Intact Cells," pages 9458-9463, see entire document, especially Table I.	1-3, 5 --- 4, 6-8
X --- Y	Biochemical Journal, Vol. 268, issued 1990, Brindle et al., "Anti-(insulin receptor) monoclonal antibody-stimulated tyrosine phosphorylation in cells transfected with human insulin receptor cDNA," pages 615-620, see entire document, especially page 617.	1-3, 5 --- 4, 6-8
Y	US, A, 4,536,517 (FLOYD, JR. ET AL.) 20 August 1985, col. 1, line 58-col. 2, line 5.	4, 6-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 JUNE 1996	Date of mailing of the international search report 03 JUL 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer C Tsang for DONNA C. WORTMAN, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04226

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US, A, 5,405,855 (ANDRULIS, JR.) 11 April 1995, col. 3, line 26-col. 4, line 52.	4, 6-8